Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titrating Tissue Culture Adapted Vaccine Strains of Infectious Bursal Disease Virus

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1. Introduction

This Supplemental Assay Method (SAM) describes a procedure using chick embryo fibroblast (CEF) cell cultures for titrating tissue culture adapted strains of virus used as vaccines against infectious bursal disease (IBD). The vaccine is composed of a preparation of the virus in a suitable stabilizer.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

2.1.3 Water-jacketed incubator with a humidified 5% CO_2 atmosphere and temperature set at 37°C (Forma Scientific, Model No. 3158)

2.1.4 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

2.1.5 Magnetic stir plate

2.1.6 Scissors, sterile (Roboz Model No. RS-6800)

2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)

2.1.8 Microliter pipette (Rainin Pipetman, P1000)

2.1.9 250-ml trypsinizing flask with stir bar, sterile

2.1.10 Hemocytometer

2.1.11 Bunsen burner

2.1.12 Blunt thumb forceps, sterile

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs

2.2.2 Tissue culture dish, 150 x 10 mm (Falcon, Cat. No. 1058)

2.2.3 Tissue culture dish, 100 x 20 mm (Falcon, Cat. No. 3003)

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29 x 114 mm, sterile, 50 ml (Sarstedt, Cat. No. 62.547.205)

2.2.6 Polypropylene centrifuge tubes, 250 ml (Corning, Cat. No. 25350)

2.2.7 Roller bottles, 1000 ml (Falcon, Cat. No. 3007)

2.2.8 Serological pipets (Falcon, Cat. No. 7530)

2.2.9 24-well tissue culture treated plate (Costar, Cat. No. 3524)

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal Bovine Serum (FBS)

2.2.12 L-Glutamine (Sigma, Cat. No. G7513)

2.2.13 Trypsin, 0.25% (Cello Corporation, Cat. No. AT25)

2.2.14 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)

2.2.15 Solutions

All solutions are filter sterilized.

1. Dulbecco's Phosphate Buffered Saline (PBS), Ca and Mg free

NaCl	8.0	g
KCl	0.2	g
Na_2HPO_4	1.03	g
KH_2PO_4	0.2	g
q.s. with distilled or deionized water	1.0	L

2. Trypsin Solution (0.25%)

NaC1	8.0	g
KC1	0.5	g
Glucose	1.0	g
Phenol Red (0.5% solution)	1.0	ml
Trypsin (1:250)	2.5	g
$NaHCO_3$	0.35	g
q.s. with distilled or deionized water	1.0	L

Adjust pH to 7.4 with $NaHCO_3$ solution.

3. Growth Medium

Medium 199 (with Earle's salts) (powdered)	9.9	g
Nutrient Mixture F1O (powdered)	9.8	g
Bacto Tryptose Phosphate Broth (powder)	2.95	g
$NaHCO_3$	2.5	g
Penicillin (potassium G)	100,000	units
Streptomycin	200	mg
Fetal Calf Serum (inactivated)	110	ml
q.s. with distilled or deionized water	1	L

Adjust pH to 7.35 to 7.4 by adding $NaHCO_3$ solution.
Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

2.2.16 Cell cultures

Primary chick embryo cell cultures are used for the titration.

Prepare primary chick embryo cell cultures from 9- to 11-day-old embryos (derived from specific-pathogen-free flocks) in the following manner: Swab the air cell end of the egg with 70% ethanol, flame, and break open the shell with sterile blunt thumb forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable petri dish. Remove (and discard) the heads of the embryos with sterile scissors. Wash the embryos in Dulbecco's PBS Ca- and Mg-free solution (see **part 2.2.15**). Open the body cavity of each embryo with the sterile forceps and remove the liver and the bulk of the other viscera. Gently squeeze the remainder of the embryo with the forceps to remove as much blood as possible. Pick the washed embryos out of the wash solution with the forceps, drain them momentarily, and place them in a sterile, dry petri dish. Mince the embryos thoroughly by cutting with sharp, sterile scissors.

Place the minced tissue in a 250-ml sterile trypsinizing flask with a magnetic stirring bar. Add 30 ml of 0.25% trypsin solution (prewarmed to approximately 35°C), and trypsinize for 5 min at room temperature. Carefully decant the supernatant suspension and discard. To the remaining fragments in the trypsinizing flask, add 70 ml of 0.25% trypsin and trypsinize for another 40 min.

Decant the cell suspension through a sterile funnel with 4 layers of gauze into a sterile container. Then filter again through 4 layers of gauze into a centrifuge bottle. Approximately 30 ml of growth medium (see **part 2.2.15**) should be added to the centrifuge bottle to stop the action of the trypsin on the cells. Centrifuge at approximately 250 X *g* for 10 min. Carefully remove the supernatant fluids. Resuspend the cells in growth medium to a concentration of approximately 800,000 cells per ml. Plant the cells in 24-well tissue culture plates, 1 ml per well. Incubate in a high humidity atmosphere containing

approximately 5% CO_2. The cultures may be inoculated any time within 4 hr after planting.

3. Preparation for the test

3.1 Personnel qualifications/training

The executor must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. The executor must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent; and training in the operation of the necessary laboratory equipment listed in **part 2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions and monitor in compliance with current corresponding CVB-L/National Veterinary Services Laboratories (NVSL) Standard Operating Procedures (SOPs) or equivalent.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 Preparation of vaccine for titration

Rehydrate vaccine in 100 ml of sterile purified water. Mix thoroughly. If necessary, further dilute the vaccine so that 1 dose is contained in a volume of 0.1 ml.

4. Performance of the test

4.1 Preparing dilutions and inoculating plates

Prepare dilution blanks of growth medium (see part **2.2.15**). Make tenfold dilutions of the vaccine encompassing the range of the expected titer. Use 4 dilutions for a titration. Inoculate 5 wells for each dilution using 0.1 ml for each well. The fifth well in each row remains uninoculated for a cell control. Incubate in a humidified atmosphere of approximately 5% CO_2 at 37°-38°C.

5. Interpretation of the test results

5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference must be within the established range for the test results to be valid. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.

5.2 Calculating the titer

Examine the plates daily, and observe for the development of areas of refractile cells typical of bursal disease virus cytopathology. The control wells must remain normal throughout the test. On day 7 postinoculation (PI), calculate the 50% endpoint of infectivity using the Reed-Muench method. This value will be the titer, since the 0.1 ml volume of inoculum per well represents 1 dose.

5.3 Retests

Conduct retests as required by the Code of Federal Regulations, Title 9 (9 CFR), part 113.8(b) and requirements of minimum release in firm’s current Outline of Production, Part V.

5.4 Evaluation of test results

5.4.1 The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

5.4.2 The firm's requirements of minimum release/stability titers for each bursal vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of test results

Titers are reported out as $TCID_{50}$ per bird dose.

7. Changes

This document was rewritten to meet the current CVB-L QA SAM format. No significant changes were made from the previous protocol. This document supersedes the September 16, 1983, version.